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SKELETAL MUSCLE GROWTH IS STIMULATED BY INTERMITTENT STRETCH/RELAXATION IN TISSUE CULTURE

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Abbreviated Title: Stretch-induced Skeletal Muscle Growth

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ABSTRACT

Avian pectoralis muscle cells differentiated _in vitro_ are mechanically stimulated by repetitive stretch/relaxation of the cell's substratum using a computerized Mechanical Cell Stimulator device. Initiation of mechanical stimulation increases the efflux of creatine kinase from the cells during the first eight to ten hours of activity, but the efflux rate returns to control levels after this time period. Decreased total cell protein content accompanies the temporary elevation of creatine kinase efflux. With continued mechanical stimulation for 48 to 72 hours, total cell protein loss recovers and significantly increases in medium supplemented with serum and embryo extract. Myotube diameters increase and cell hyperplasia occurs in the stimulated cultures. In basal medium without supplements, mechanical activity prevents myotube atrophy, but does not lead to cell growth. Mechanically-induced growth is accompanied by significant increases in protein synthesis rates. The increases in protein synthesis and accumulation induced by mechanical stimulation are not inhibited by tetrodotoxin but are significantly reduced in basal medium without supplements. Mechanically-stimulated cell growth is thus dependent on medium growth factors but independent of electrical activity.

Index Terms: Skeletal muscle; growth; protein synthesis; creatine kinase; electrical activity; mechanical activity; growth factors.

INTRODUCTION

Skeletal muscle use is a major determinant in regulating protein turnover rates and growth of the tissue. Increased usage leads to tissue growth while disuse causes atrophy. Tension development in the muscle fibers associated with increased activity is a major regulator of muscle growth in embryonic, neonatal, and adult skeletal muscle (reviewed in 27). The molecular mechanism by which physical stimuli such as muscle tension is transduced into the biochemical alterations associated with muscle growth is unknown. A number of _in vivo_ and _in vitro_ model systems are used to study the morphological and biochemical processes of tension-induced skeletal muscle growth. The _in vivo_ models include tenotomy of synergistic muscle (12), casting of limbs such that the muscle is held in a stretched (i.e. increased tension) position (14,17), and hanging weights on a limb (22). All of these model systems appear to stimulate muscle growth by stretching of the muscle fibers. In organ culture, stretching of muscle fibers also stimulates the biochemical processes associated with _in vivo_ muscle growth such as increased sodium-dependent amino acid transport (13), glucose uptake (21), metabolic rates (9) and protein synthesis (3,21). While these _in vitro_ organ culture systems are excellent for short term biochemical studies, they are inappropriate for long term growth studies because the isolated muscles are in negative nitrogen balance and survive only a short time _in vitro_ (27).

Tissue cultured muscle cells are extensively used to study the differentiation and development of skeletal muscle under defined *in vitro* conditions. Their *in vitro* development closely follows *in vivo* growth in many respects, including the formation of multinucleated contractile myotubes and the progressive increase in the synthesis and organization of muscle specific proteins (23). We have shown previously that cultured muscle cells respond to passive stretch in a manner very similar to *in vivo* and organ cultured skeletal muscle (26,30,31,33). In this paper, we describe the growth response of muscle cells differentiated in tissue culture to repetitive intermittent stretch/relaxation activity for 2-4 days. Mechanically-induced cell growth occurs in this model system. This results from an increase in protein synthesis rates in the cells which is dependent on medium growth factors but independent of electrical activity in the cells. This model system should complement *in vivo* and organ cultured systems to better understand the molecular mechanism by which muscle tension is transduced into the biochemical alterations associated with muscle growth. Parts of this work have appeared in abstract form[1].

MATERIALS AND METHODS

Cell Cultures

Avian myoblasts are isolated from 11-12 days *in ovo* pectoralis muscle by standard dissection techniques (29). Fertilized chicken eggs are obtained from Beaver River Farm, W. Kingston, RI. The cells are plated at a density of 5,700 cells/mm^2 in 1 ml of growth medium and maintained in a humidified 5.0% CO^2 incubator at 37.2°C. Growth medium is Eagle's Basal Medium containing 50 units/ml penicillin (Sigma Chemical Co., St. Louis, MO), 10% horse serum (Hyclone Laboratories, Logan, UT), and 5% chicken embryo extract (85/10/5 medium). Cells are plated and grown on a collagen-coated elastic substratum in a 24 well growth chamber of a Mechanical Cell Stimulator (Model 1) device described previously (28). The cultures are fed 1 ml of fresh 85/10/5 medium every 24 hours. Under these growth conditions, the myogenic cells proliferate and fuse during the first 24-72 hours *in vitro*, become striated and highly contractile by 96-120 hours and remain so for the duration of the experiments. Growth and differentiation of the muscle cells in the wells of the Mechanical Cell Stimulator are similar to collagen-coated wells in commercial tissue culture dishes (28).

At 48-54 hours postplating the cells are embedded in a collagen gel matrix (29). This embedding technique allows mechanical stimulation of the myotubes for extended periods without rupturing or detachment[1]. Briefly, the culture medium is removed from the cells, and 0.5 ml of ice cold collagen solution added. The collagen solution is prepared immediately before use by mixing 85/10/5 medium with rat tail collagen (Type I, Collaborative Research, Bedford, MA) to a final concentration of 200 to 400 μg/0.5 ml. Sterile sodium hydroxide (0.1 N) is added to the chilled solution to maintain a pH of 7.0. Stainless steel wire cloth (Newark Wire Cloth, Newark, NJ) is placed inside the perimeter of each well immediately after the collagen gel solution is added to the wells but before its polymerization. The screen acts as an "artificial tendon" by providing a surface to which the differentiating cells and collagen gel can attach and be held in position during long term repetitive mechanical stimulation. Culture medium (85/10/5) is added to the top of the hardened collagen gel 24 hr after solidifying, and changed every 24 hr thereafter. After several days the collagen gel dehydrates, collapsing onto the muscle cells as a thin, 12 to 50 μm thick layer on top of the cells (29) in the same manner as described for other cell types (5).

Mechanical Stimulation

The Mechanical Cell Stimulator (Model 1) used to repetitively stretch and relax the culture substratum and the attached muscle cells has been described previously (28). Briefly, a Cell Growth Chamber containing 24 15 mm diameter wells is secured above a moveable aluminum platform containing 12 2 mm wide by 11 mm high aluminum prongs which center on 12 of the culture wells. A Stepper motor moves the prong platform up and down in 35 μm increments causing the prongs to stretch and relax the elastic substratum and attached muscle cells (27,28). The prongs are removable from the apparatus and prongs of different heights can be used to vary the total percent stretch of the substratum during the same experiment. For example, a 2 mm vertical prong movement equals 3.5% substratum stretch and a 5 mm vertical prong movement equals a 20.0% substratum stretch (28). The percent stretch used in the experiments described in this paper varies from 3.5 to 46%. The activity pattern of the Mechanical Cell Stimulator is controlled by an Apple IIe computer connected to the stepper motor.

Mechanical activity program TRIAL39.PGM (Figure 1) is used to repetitively stretch and relax the substratum on which the cells grow. This program consists of 5 substratum stretches and relaxations during a 20 second period followed by a 10 second rest; this activity is repeated three times, followed by a 30 minute rest period. The cells are thus mechanically stimulated for a total of 60 seconds every thirty minutes (i.e. 3.3% of experiment time). The rate of stretch and relaxation is 2.5 mm/sec. Unstimulated controls are maintained in the same growth chamber as the cells which are mechanically stimulated. Mechanical activity is begun on Day 6 to 8 postplating, when the myotubes are well differentiated and structurally strong enough to withstand this repetitive activity, based on cell morphology and creatine kinase release (see Results).

Figure 1.

Biochemical Assays

Creatine kinase activity released from the cells into the culture medium is measured with a commercially available assay kit (Procedure No. 47-UV, Sigma Chemical Co., St. Louis, MO). An aliquot (20 μl) from the medium is mixed with 1 ml creatine kinase reagent and the absorbance at 340 nm caused by NADH production is followed for 5 min at 30°C. The change in rate of absorbance is directly proportional to creatine kinase activity. Medium activity is stable for up to 14 days at 4°C (data not shown).

Protein synthesis is measured with ^{14}C-phenylalanine incorporation into trichloroacetic acid insoluble material. At varying times, culture medium is replaced with 1 ml/well Eagle's Basal Medium with glutamine (2 mM), penicillin (50 units/ml), 0.6 mM nonradioactive phenylalanine, and 1μCi/ml ^{14}C-phenylalanine (L-[U-14-C] phenylalanine, Amersham, Arlington Heights, IL) for 1 to 4 hr at 37°C on rotary shaker (60 rpm). Using excess nonradioactive phenylalanine in the medium gives an accurate indication of protein synthesis rates in cultured muscle cells without alterations in the free intracellular phenylalanine pool (32). After incubation the cells are removed from the Mechanical Cell Stimulator, transferred with forceps to 2 ml ice cold Earle's Balanced Salt Solution (phenol red free) in 35 mm petri dishes on ice, and shaken at 120 rpm for 60 min with solution changes every 10-15 min. The rinsed cells are sonicated in 0.5 ml ice cold sucrose buffer (0.25 M sucrose, 0.02 M KCl, pH 6.8) and aliquots removed for determination of total noncollagenous protein, total DNA, and trichloroacetic acid soluble and insoluble radioactivity. Noncollagenous proteins are extracted by the method of Lowry et al. (20) and protein determined by the BCA Protein Assay (Pierce Chem. Co., Rockford, IL). Total DNA is determined fluorometrically as described previously (29). An aliquot of the sonicate is made 5% (v/v) with ice cold trichloroacetic acid. After 20-30 min on ice, the sonicate is centrifuged (3,000 xg) at 4°C for 10 min and the radioactivity in the supernatant (trichloroacetic acid soluble radioactivity) measured with a Packard 450C Scintillation Counter. The precipitate is rinsed 3 times with 5% (v/v) trichloroacetic acid, the pellet dissolved in 0.1 N NaOH, and radioactivity measured (trichloroacetic acid insoluble radioactivity). The protein synthesis rates are expressed as either trichloroacetic acid insoluble radioactivity/trichloroacetic acid soluble radioactivity per μg noncollagenous protein in incorporation analyses of less than 4 hrs or as trichloracetic acid insoluble radioactivity per μg noncollagenous proteins for longer incorporation periods. Tetrodotoxin (Sigma Chemical Co., St. Louis, MO) is used at 3 μg/ml and added to the cultures at least 1 hr before the start of mechanical stimulation. This concentration completely inhibits spontaneous muscle contractions as observed by inverted phase microscopy.

Statistical analysis of the biochemical measurements is performed by t-tests for paired and unpaired values (PC

Statistician Software, Human Systems Dynamics, Northridge, CA).

Morphometry

The culture wells are placed on ice, rinsed with ice cold phosphate buffered saline (Earle's Balanced Salt Solution, phenol red free) and fixed in ice cold ethanol: acetic acid (3:1, v/v) for at least 10 min. The cells are stained with Harris' hemotoxin/eosin or toluidine blue and embedded in Spurr Embedding Medium (Polysciences, King of Prussia, PA). A water-soluble embedding medium such as Spurr is required to prevent elastic substratum distortions which occur if the substratum is exposed to organic solvents. Following hardening of the embedding medium overnight at 70°, the translucent substratum is carefully removed from the well bottoms and the blocks containing the stained cells are mounted upside down on a glass slide. With these whole mounts, the cells can be clearly observed with a light microscope (10x to 100x oil immersion objective, total magnification of 125 to 1250x).

Microscope fields are observed using a Zeiss microscope equipped with a drawing tube attachment focused onto a Numonics 2210 Digitizing Table connected to a Compaq Deskpro 286 computer. The stained cells and tablet mouse can be seen while looking through the microscope eyepieces. Morphological measurements are made using morphometry software (SIGMA SCAN TM, Jandel Scientific, Sausalito, CA). All data is computer corrected for magnification.

Mean myotube diameters are measured on randomly selected fields. The length and area of every myotube in the field is measured and the mean diameter calculated. In preliminary experiments, thick sections were cut from the Spurr-embedded blocks (Porter Blum MT2B microtome) and cross sectional diameters of the myotubes measured. Measuring the mean myotube diameters in whole mounts from both control and stretched cultures gives similar values to cross sectional diameter measurements (data not shown). All measurements are made independently by two or three individuals on samples from two separate experiments. Similar results were obtained by the different observers.

Myosin Heavy Chain Staining

To visualize the network of muscle cells which are mechanically stimulated, 6 to 8 day postplating myotubes are stained by enzyme immunoassay. A monoclonal antibody specific for avian embryonic fast myosin heavy chains (EB165) is used (7) together with avidin-biotinylated secondary antibody reaction coupled to horse radish peroxidase (Vectastain ABC, Vector Laboratories, Burlingame, CA).

RESULTS

Under appropriate tissue culture conditions, embryonic skeletal myoblasts rapidly develop into a dense two dimensional network of interconnected multinucleated myotubes (Figure 2A). These myotubes are spontaneously contractile and well-striated by 6 days in vitro (Figure 2B). Interspersed between these myotubes are mononucleated fibroblasts. These differentiated myotubes are encased in a three dimensional collagen gel matrix (29). Embedded in the collagen gel, the myotubes develop more extensive myofibrillar and external lamina structures (29). This gives the cells the structural strength to withstand repetitive stimulation without extensive damage[1]. Without collagen embedding, the myotubes rupture and detach after 12-24 hr of mechanical stimulation. The resultant collagen embedded tissue is similar to a diaphragm in gross morphology. The Mechanical Cell Stimulator device in which the cells grow is used to mechanically stretch and relax in two dimensions the substratum on which the cells grow and thereby stretches and relaxes the cells (27,30). We have previously shown that the attached myotubes are longitudinally stretched and relaxed to the same percent as the substratum (30). The percent of substratum stretch used to stimulate the cells in the present experiment ranged from 3.5% to 46%. The cells are mechanically stimulated for only 3.3% of incubation time in all experiments (Figure 1). No reorientation of the stimulated myotubes has been noted by inverted phase microscopy in these experiments.

Figure 2.

Creatine kinase efflux from the cells increases when mechanical stimulation is initiated and the efflux rate activity is proportional to the percent of substratum stretch (Figure 3). Unstimulated control cultures also display an elevation in creatine kinase efflux 5 to 10 hr after the start of the experiment, probably reflecting the switch in the cells growth medium at the start of the experiment from one containing serum and embryo extract to one free of supplements. Protein degradation rates are elevated substantially in muscle cultures when serum and/or embryo extract is removed from the medium (31) and this increase may be related to elevated creatine kinase efflux in the controls. With continued mechanical activity beyond 8-10 hr, creatine kinase efflux decreases and returns to control levels (20% stretch/ relaxation) by 24 hr and remains similar to control levels for the next 72 hr of mechanical stimulation (Figure 3). The stretch-induced efflux of creatine kinase from the muscle cells may result from either irreversible damage to a few of the myotubes in the culture, or reversible damage to the cells.

Figure 3.

Figure 4.

Total cellular protein content of the cultures decreases by 22% during the first 6 hr of initiating mechanical activity (Figure 4), correlating with the increase in creatine kinase efflux during this period. With continued activity, this protein loss is recovered (Figure 4) and in medium supplemented with serum and embryo extract, the stimulated cells continue to accumulate protein over the next 38-40 hr, so that by 48 hr of activity, the cultures contain 24%

more protein than unstimulated controls (Figure 4, solid lines). In unsupplemented medium (no serum or embryo extract), mechanical activity helps to prevent the protein loss which occurs in unstimulated control cultures (Figure 3, dashed lines) but these cultures never accumulate protein to a greater extent than present at the start of the experiment. Results similar to these have been obtained in over 12 separate experiments.

In addition to stimulating protein accumulation, mechanical activity also stimulates cell proliferation, indicated by a rapid increase in the DNA content of the stimulated cultures (Figure 5). Similar results have been obtained in 6 different experiments. This stimulation of cell proliferation occurs to an equal extent in complete and basal medium (Table I), even though total DNA content of unstimulated control cultures decreases by 55% when maintained for 2 days in basal medium (Table I). This decrease in total control culture DNA content in basal medium results from an equal decrease in myotube and mononucleated cell nuclei densities (data not shown). Hyperplasia is thus a consistent feature of mechanical stimulation of the cultured cells in media of various compositions.

Figure 5.

Table 1.

Skeletal myotubes differentiated in tissue culture display a wide range of diameters, from less than 5 μm to greater than 55 μm. To determine whether mechanical stimulation of the tissue culture cells causes myotube hypertrophy as well as cell hyperplasia, a large number of myotube diameters were measured to account for the wide range of control values. In preliminary experiments, cross sections were cut from control and stimulated cultures and myotube diameters measured. In the same cultures, mean myotube diameters were calculated in whole mounts as outlined in Materials and Methods. Both methods gave similar results (Data not shown). Since the later method is more convenient for large numbers of measurements, and is not subject to the problem of cutting cross sections perpendicular to the long axis of the myotubes, it was used to analyze whether myotube hypertrophy occurs. Mechanical stimulation of the cultures for 2 to 4 days in complete medium (10% serum, 5% embryo extract) results in a significant 16% increase in mean myotube diameter from 19.2 μm in control cultures to 22.3 μm in the stimulated cultures (Figure 6A). If the cultures are maintained for 2 to 4 days in basal medium (no supplements), mean control myotube diameter decreases from 19.2 to 17.2 μm (Figure 6B). Mechanical stimulation of these cultures prevents this decrease in mean myotube diameter (Figure 6B). These results compare well with the total cell protein accumulation data in complete versus basal medium (Figure 4). Mechanical activity thus appears to cause myotube hypertrophy in complete medium and prevent myotube atrophy in basal medium.

Figure 6.

Figure 7.

Protein synthesis rates in the cultures increase significantly within 3 hr of initiating mechanical activity in complete medium (Figure 7). By 24 hr of activity, the protein synthesis rate is increased 30%, 97% by 48 hrs, and 71% by 96 hrs (Figure 8). Mechanically stimulating the cells in basal medium without supplements for 2 days also stimulates protein synthesis rates compared to controls, but the percent stimulation is reduced by 55% (Table II). This compares to a 39% decrease in total cell protein content of the mechanically stimulated cultures in complete versus basal medium (Figure 4).

Figure 8.

Table 2.

Protein accumulation and synthesis rates increase significantly in complete medium when the percent stretch is greater than 5% (Figure 9). Protein accumulation is similar at 8, 13, and 20% stretch/relaxation, but protein synthesis is optimally stimulated at 8% stretch/relaxation when compared to the increases at 13 and 20%. The intensity of stretch/ relaxation thus influences the growth response of the cells even though the pattern of activity remains the same (Figure 1).

Figure 9.

Repetitive mechanical stimulation of the muscle cultures might stimulate muscle cell growth by altering spontaneous contractile activity in the tissue cultured cells. To determine the importance of electrical activity for mechanically-induced protein synthesis and accumulation, tetrodotoxin was used to inhibit the voltage-sensitive sodium channels of the muscle cells. Mechanical stimulation of the cultures for 2 days in complete medium containing tetrodotoxin prevents spontaneous activity as observed by inverted phase microscopy, but had no effect on mechanical stimulation of either protein synthesis or accumulation (Table III). Electrical activity, therefore, is not essential for the growth response of the cells to mechanical stimulation.

Table 3.

DISCUSSION

The major finding in this study is that a diaphragm-like two dimensional network of tissue cultured skeletal myotubes differentiated from embryonic mononucleated myoblasts can be mechanically stimulated to undergo both hypertrophy and hyperplasia by intermittent stretches and relaxations. This computerized Mechanical Cell Stimulator model system thus provides a new method for the analysis of skeletal muscle growth in response to mechanical activity. The system offers a number of advantages over current _in vivo_ and _in vitro_ methods for studying stretch-induced skeletal muscle growth (reviewed in 27). These include the ability to better control the cells' external environment with regard to growth factors, the ability to keep the muscle cells in positive nitrogen balance for extended periods (i.e. weeks), and the ability to study biochemical alterations in the muscle cells separate from the influences of vascular or nerve tissue. The principal disadvantage of the culture system is that the muscle cells are not adult fibers, but are neonatal-like myotubes based on myosin heavy chain isoforms (7) and structural criteria (29). The neonatal myotube's response to mechanical stimulation may therefore not be identical to adult muscle cells.

The cultured myotubes in this study originate from pectoralis myoblasts which _in vivo_ differentiate into fast glycolytic myofibers. The pattern of mechanical activity chosen for this study (Figure 1) is of moderate intensity

(20% stretch/relaxations) and infrequent duration, with the cells stimulated for 60 seconds every 30 minutes. This intermittent pattern was chosen to represent the functional demands which might be placed on neonatal muscle <u>in vivo</u>. This pattern of activity has been found to increase the cell's glycolytic rate several-fold (16). Other patterns of activity have been used to inhibit glycolysis and stimulate oxidative metabolism in the cells[2]. The tissue culture model system thus provides a method of studying the relationship of mechanically-induced metabolic alterations to muscle growth.

Temporary damage is done to the muscle cells when mechanical stimulation is initiated, based on the elevated release of creatine kinase into the culture medium within 2 hrs of starting the activity (Figure 3). Increasing the intensity of the stimulus from 20% to 46% stretch/relaxation activity causes a significant 84% increase in the efflux of creatine kinase (Figure 3). These alterations are similar to the elevated serum creatine kinase activities which occur following exercise in untrained individuals (19) and to the direct relationship <u>in vivo</u> between the intensity of exercise and the level of released enzyme activity (25). After 20 hrs of continued activity in tissue culture, creatine kinase efflux rates return to control levels and remain at these levels for the duration of the experiments. The mechanism for this adaptive response in the cultured system is not known. Reduced creatine kinase efflux occurs in trained muscles <u>in vivo</u> (11) and it is interesting to postulate that a common mechanism might be involved.

The dual stretch response of skeletal muscle hypertrophy and hyperplasia in the tissue culture model system is similar to that which occurs in most in vivo stretch-induced muscle growth models. These include functionally overloaded skeletal muscle (15), stretch-immobilized skeletal muscle (14), and passively stretched skeletal muscles (17). The hyperplasia in these in vivo muscle models results from increased vascular and connective tissue cell proliferation but this hyperplastic response to stretch is not essential for adult myofiber hypertrophy in vivo (10). Whether mechanical stimulation in tissue culture increases the proliferation rate of fibroblastic connective tissue cells only, or also stimulates myogenic proliferation is not presently known. Longitudinal myotube growth in tissue culture induced by mechanical stretch involves the stimulation of fusion of myogenic cells into the growing myotubes[3] and it has been recently shown that exercising skeletal muscle in growing animals leads to a large mitogenic response of the myogenic population (8).

The mechanism by which mechanical activity stimulates hyperplasia is not known. Myogenic cells are contact inhibited to a certain extent in tissue culture (1), although fibroblasts do overgrow differentiated myotubes. As the cells become more confluent in vitro their growth rate decreases (28). Mechanical stretching releases the cells from this inhibition and allows them to proliferate[3]. Endogenous growth factors may be synthesized and released at increasing rates from the mechanically stimulated cells and these factors could stimulate cell proliferation. Such factors have been found in tissue-cultured myotubes (26), adult skeletal muscle (18,34), and in skeletal muscle from young animals undergoing stretch-induced hypertrophy (24).

Mechanically-induced myotube hypertrophy occurs in the tissue culture model system as indicated by the 16% increase in mean myotube diameter. This response requires some as yet undefined factor present in serum and/or embryo extract. It is not known whether this hypertrophic response is coupled to the proliferative response of the cells to mechanical activity. In basal medium without supplements, cell proliferation is reduced by 55% compared to growth of the cells in supplemented medium (Table III), but mechanical activity is still able to significantly stimulate cell proliferation to the same extent as in supplemented medium (Table III). Mechanical activity in basal medium does not lead to myotube hypertrophy but does prevent myotubes from atrophying. Whether the differences in the hypertrophic and hyperplastic growth response to mechanical activity in the various media are inter-related is not presently known.

Mechanically-induced cell growth in this model system results from large and sustained increases in protein synthesis rates (Figures 7 and 8). The stretch-induced increase in protein synthesis is partially dependent on medium growth factors (Table 2) since the percent increase is reduced in basal medium without supplements. In contrast, stretch-induced increases in protein synthesis rates are independent of electrical activity since tetrodotoxin is without effect (Table 3). These latter results are in agreement with those of Bandman and Strohman (4) which show that total muscle protein synthesis in tissue cultured cells is independent of spontaneous contractile activity. The rate of contractile protein degradation in these cells is highly dependent on spontaneous electrical activity (4). Whether mechanical stimulation alters contractile protein degradation rates in an electrical activity-dependent manner is currently under investigation.

In summary, a dynamic tissue culture model system has been developed which mimics many of the characteristics of stretch-induced skeletal muscle growth <u>in vivo</u>. Mechanical stimulation of an intermittent type over several days causes myotube hypertrophy and cell hyperplasia under appropriate growth conditions. Medium growth factors found in serum and/or embryo extract affect the growth response of the cells to mechanical stimulation. Electrical activity of the cells is not important for the stretch responses to occur. As a greater number of experiments are performed using different patterns of activity in this <u>in vitro</u> system, a better understanding of how physical stimuli such as stretch are transduced into the biochemical processes associated with growth will be possible. Combining these results with those obtained using <u>in vivo</u> and organ-culture models should eventually lead to a more complete understanding of these important biochemical processes.

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TEXT FOOTNOTES

1. Hatfaludy, S., J. Shansky, and H.H. Vandenburgh. Can. J. Sport Sciences 13, 15P Abstract (1988).

2. Vandenburgh, H.H. et. al., In Preparation.

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TABLE 1. MECHANICAL STIMULATION OF CELL PROLIFERATION IN MEDIUM OF VARIOUS COMPOSITIONS

	Group	DNA Content (μg/well)	Δ	p
A. Complete Medium (10% serum, 5% embryo extract)	Control	13.65 ± 0.77	-	
	Stretch	17.15 ± 2.05	26%↑	.05
B. Minimal Medium (2% serum)	Control	8.25 ± 0.57*	-	-
	Stretch	9.40 ± 1.21	14%↑	NS
C. Basal Medium (no supplements)	Control	6.20 ± 0.79**	-	-
	Stretch	7.45 ± 0.25	20%↑	.03

Muscle cultures 6 days postplating are rinsed 3 times rapidly and then incubated for 2 hrs at 37° with the appropriate medium. Fresh medium is added and mechanical stimulation (20%, Figure 1) initiated for 2 days with a medium change at 24 hr. Each value is the mean ± S.D. for 4 to 6 cultures and statistical analysis is by t-tests for unpaired samples.

* Control A vs. Control B, $p < .001$

**Control A vs. Control C, $p < .001$

TABLE 2. MECHANICAL STIMULATION OF PROTEIN SYNTHESIS IN MEDIUM OF VARIOUS COMPOSITIONS

	Group	14-C Phenylalanine Incorporation (TCA ppt dpm/μg protein)	Δ	p
A. Complete Medium (10% serum, 5% embryo extract)	Control	8.27 ± 0.54	-	-
	Stretch	13.55 ± 1.73	64%↑	.002
B. Minimal Medium (2% serum)	Control	10.21 ± 1.38	-	-
	Stretch	13.88 ± 0.56	36%↑	.009
C. Basal Medium (no supplements)	Control	10.53 ± 0.13	-	-
	Stretch	13.54 ± 0.80	29%↑	.002

See legend to TABLE 1 for experimental protocol. Protein synthesis rates are measured as outlined in MATERIALS AND METHODS for the last 4 hr of the experiment. Each value is the mean ± S.D.of 4 to 6 cultures and statistical analysis is by t-tests for unpaired samples.

TABLE 3. MECHANICALLY-STIMULATED PROTEIN SYNTHESIS AND PROTEIN ACCUMULATION ARE NOT INHIBITED BY TETRODOTOXIN

Group	Tetrodotoxin	Total Noncollagenous Protein (μg/well)	Δ	p	14-C Phenylalanine Incorporation (TCA ppt dpm x 10^4/well)	Δ	p
CONTROL	-	269 ± 25			4.03 ± 0.43		
STRETCH	-	379 ± 31	37%↑	.01	6.91 ± 0.40	71%↑	.003
CONTROL	+	219 ± 9			3.13 ± 0.37		
STRETCH	+	305 ± 14	39%↑	.001	7.44 0.35	138%↑	.001

Muscle cultures 6 days post-plating are intermittently stretched and relaxed (20%) for two days according to the program shown in FIGURE 1. The medium contained 10% horse serum and 5% embryo extract with (+) or without (-) 3 μg/ml tetrodotoxin. This concentration of tetrodotoxin inhibits spontaneous muscle contractions due to its inhibitory effect on the voltage-sensitive sodium channel. Each value is the mean ± SD of 4 to 6 cultures and statistical analysis is by t-tests for unpaired values.

FIGURE LEGENDS

Figure 1. Schematic diagram of stretch/relaxation program (TRIAL39.PGM) started on Day 6-7 postplating. This activity is continued for the duration of each experiment. The elastic substratum is stretched and relaxed 20% (5 mm) in 12 wells of the Mechanical Cell Stimulator while 12 control wells are not mechanically stimulated. Mechanical stimulation occupies 2 min out of every hour of incubation.

Figure 2. Two dimensional network of myotubes which are mechanically stimulated. Day 6 - Day 8 muscle cells have been stained with a monoclonal antibody specific for avian embryonic fast myosin heavy chain (EB165) coupled to an avidin-biotinylated secondary antibody and horseradish peroxidase. Fibroblasts and collagen fill the inter-muscular space, but are not visualized by this staining technique. (A) Low power magnification (100x) of cultures showing unoriented multinucleated muscle cells; (B) Higher power magnification (1500x) showing that the myotubes are well striated.

Figure 3. Creatine kinase efflux temporarily increases with mechanical stimulation. Day 6 skeletal muscle cultures are mechanically stimulated by the pattern shown in Figure 1. Stretch/relaxation activity is started at 0 time in Eagle's basal medium with glutamine and continued for up to 4 days. The 20% and 46% stretch/relaxation activity result from 5 and 8 mm vertical movement of the substratum, respectively. An aliquot of the medium is assayed for enzymatic activity at

different times after initiating activity. Each point is the mean ± S.D. of 6 to 8 cultures and statistical analysis by t-test for paired samples.

Figure 4. Total protein content of the cultures initially decreases during mechanical stimulation, but significantly increases by 48 hrs of continuous activity. Twenty percent stretch/relaxations are performed by the program shown in Figure 1 in medium containing 10% horse serum, 5% avian embryo extract (solid lines) or in basal medium without supplements (dashed lines). Mechanical activity increases protein content compared to static controls in both media, but growth occurs only in complete medium. In basal medium mechanical activity decreases the rate of protein loss from the cells. Each point is the mean ± SEM of 6 to 13 measurements from four separate experiments and statistical analyses are by either unpaired (2 hr) or paired (48 hr) t-tests. Points without error bars have SEM less than point size.

Figure 5. DNA content of the cultures rapidly increases with mechanical stimulation. Day 6 cultures are stimulated 20% starting at 0 time in complete medium (10% serum, 5% embryo extract). Each point is the mean ± SEM of 4 to 6 cultures and statistical analysis is by paired t-test for the 0.5 to 4 days of activity.

Figure 6. Mechanical activity increases myotube cross-sectional diameters in both (A) complete medium (10% horse serum, 5% embryo extract) and (B) basal medium (no supple-

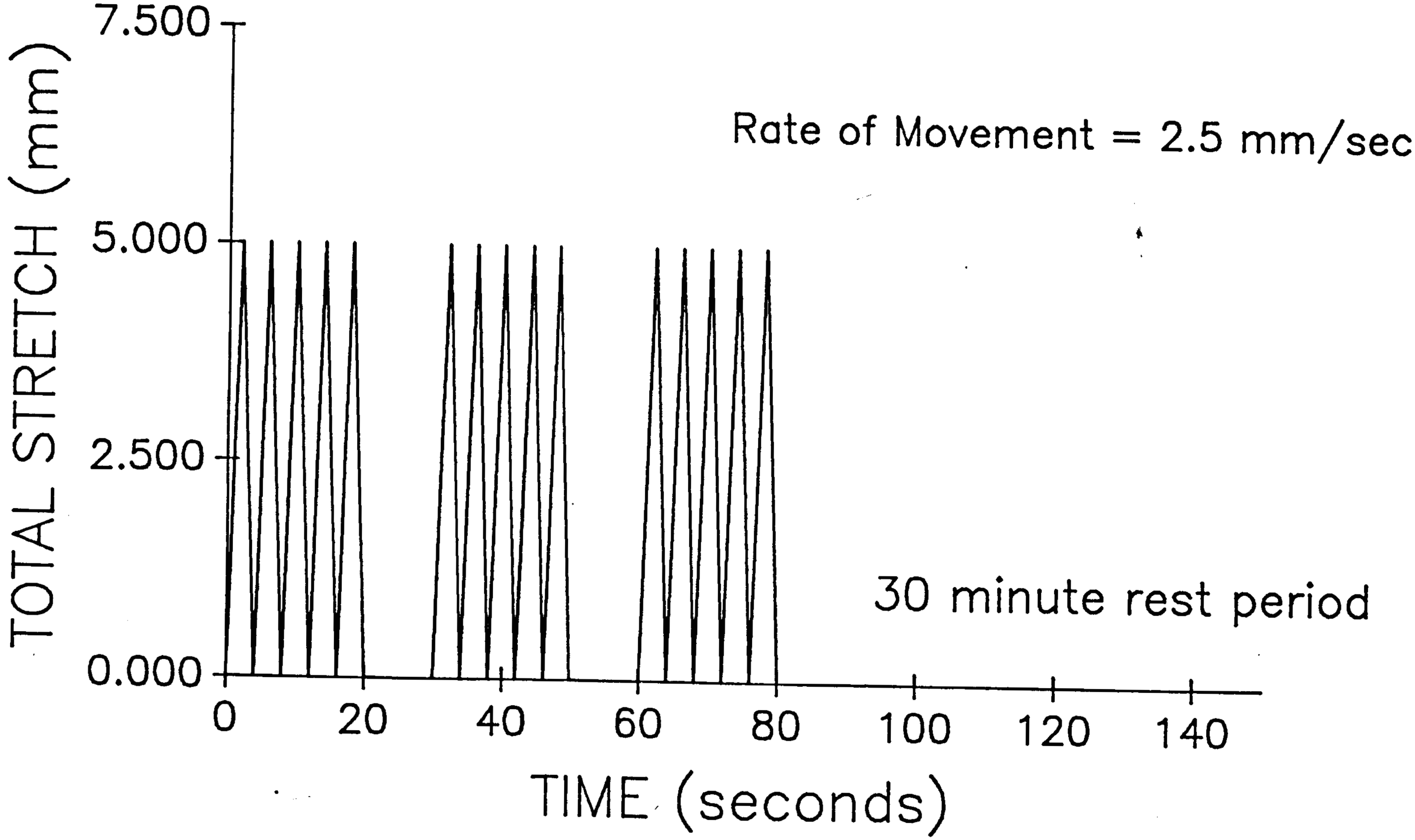
ments). Twenty percent stretch/relaxations are performed by the activity pattern shown in Figure 1 for 2 to 4 days. In complete medium (A), mean myotube diameter increases by 16% ($p < .003$) while in basal medium (B), mechanical activity prevents a 10% decrease ($p < .015$) in mean control myotube diameter. The mean ± SEM of N measurements are given in the figure and statistical analyses are by unpaired t-tests.

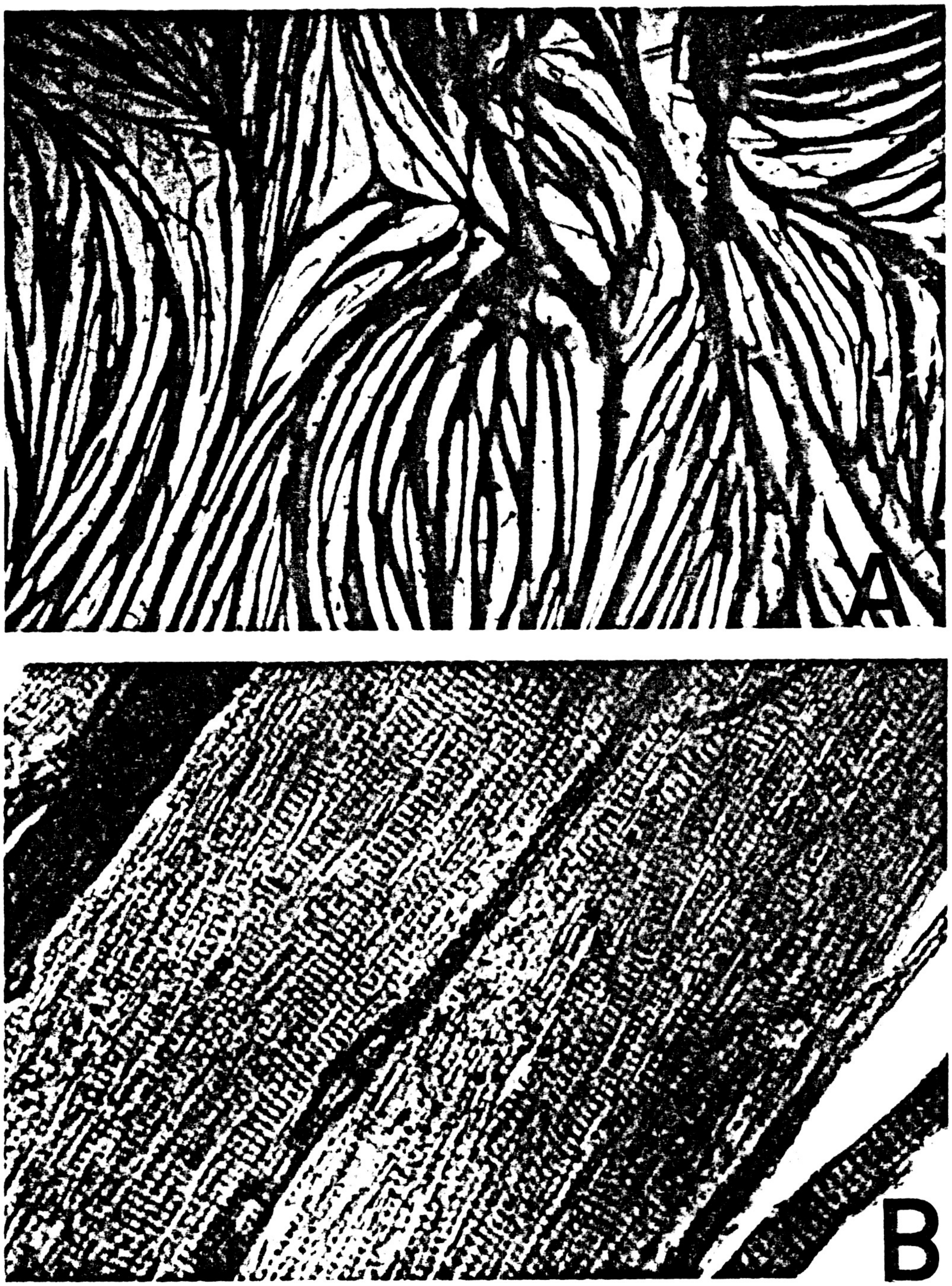
<u>Figure 7.</u> Protein synthesis is stimulated within 3 hr of initiating intermittent stretch/relaxation of the cultures. Six days after plating, fresh medium containing 1 μCi/ml 14-C-phenylalanine is added to the cultures and mechanical activity started (20% stretch/relaxation). At hourly intervals, the cells are processed for trichloroacetic acid soluble and insoluble radioactivity. Each point is the mean ± SD of 4 to 6 cultures and statistical analysis is by unpaired t-test.

<u>Figure 8.</u> Protein synthesis rates are elevated for at least 96 hr in the mechanically-stimulated cultures. Mechanical stretch/relaxation (20%) of the cells starts six days post-plating. At 24, 48, and 96 hrs the cells are pulsed for 4 hrs with 1 μCi/ml 14-C-phenylalanine and the incorporation into trichloroacetic acid insoluble protein assayed. Each bar represents 4 to 8 samples and statistical analysis is by unpaired t-tests. Each time point represents separate experiments. The mean increase in protein synthesis rates induced by mechanical activity in seven experiments of this type was 78% ($p < .001$).

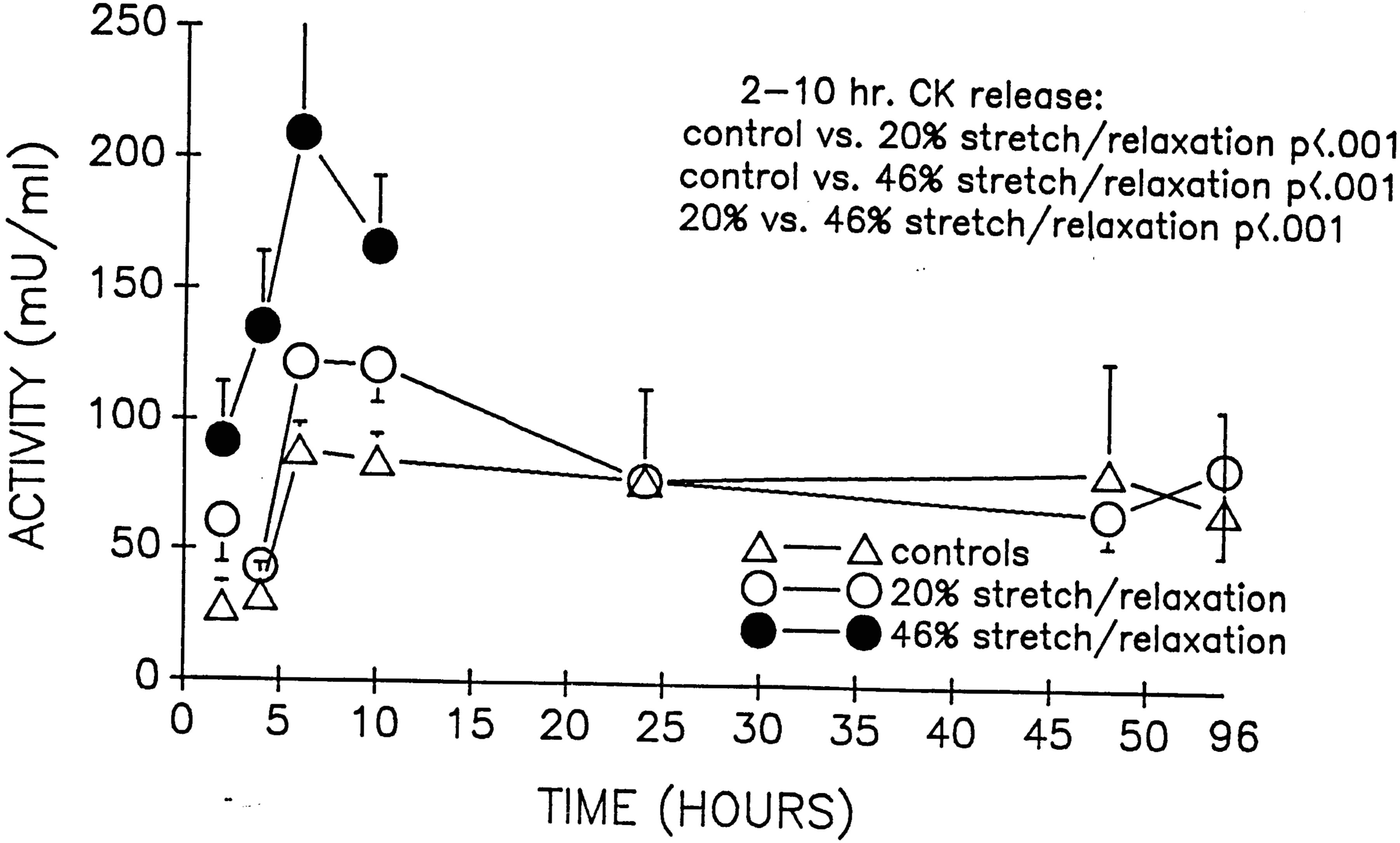
<u>Figure 9.</u> Mechanical stimulation of protein synthesis and accumulation at different percent stretch/relaxation activity. Day 6 cultures are mechanically stimulated for 2 days by the pattern of activity shown in Figure 1, but the percent stretch is varied from 3.5 to 20% (1 mm to 5 mm). During the last 4 hr of incubation, protein synthesis rates are measured as outlined in MATERIALS AND METHODS. Each point is the mean ± S.D. of 4 to 6 cultures and statistical analysis is by t-test for unpaired samples.

REPETITIVE STRETCH PROGRAM
(TRIAL39.PGM)

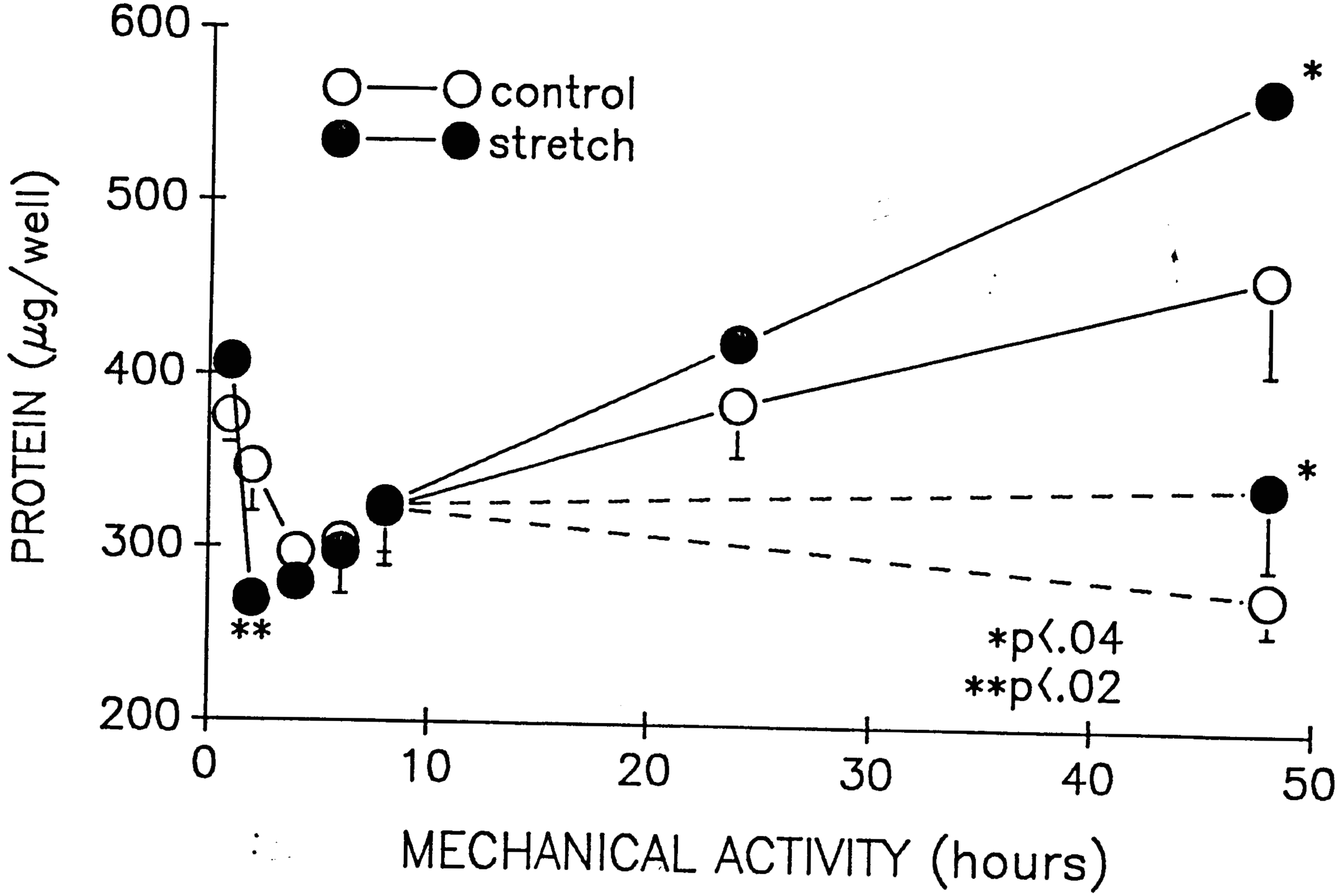



A
B

CREATINE KINASE RELEASE IN BASAL MEDIUM

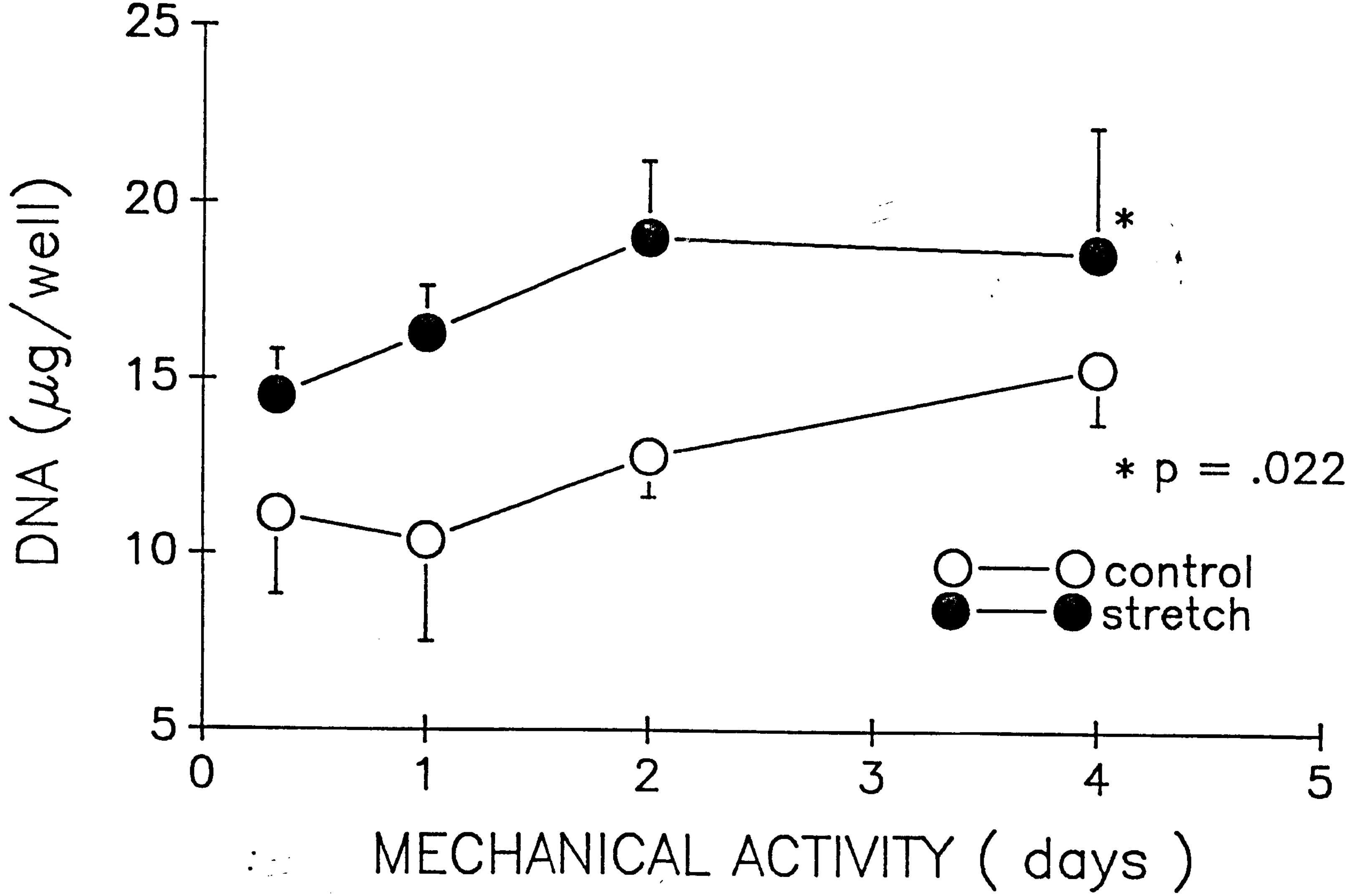


TOTAL PROTEIN

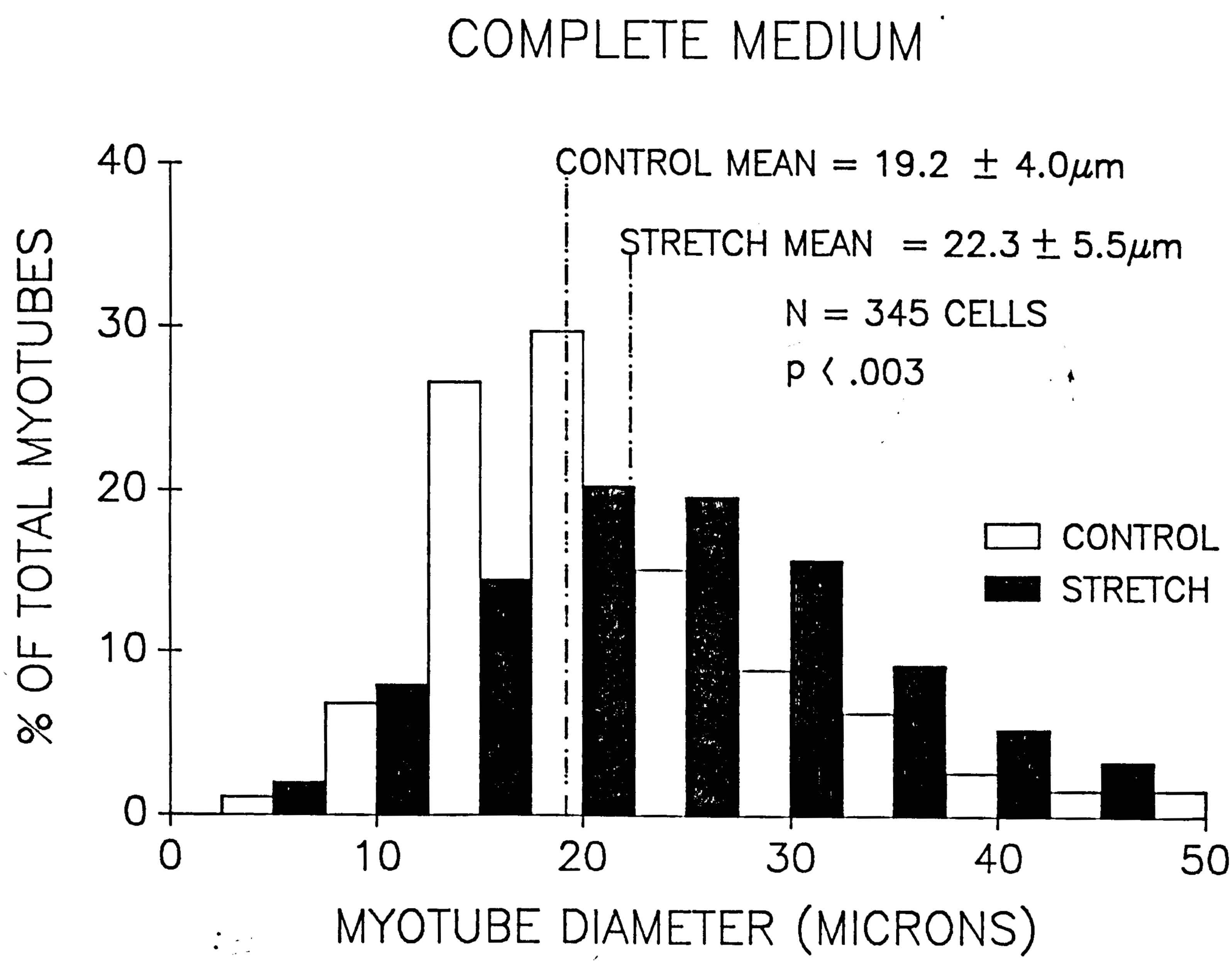


5

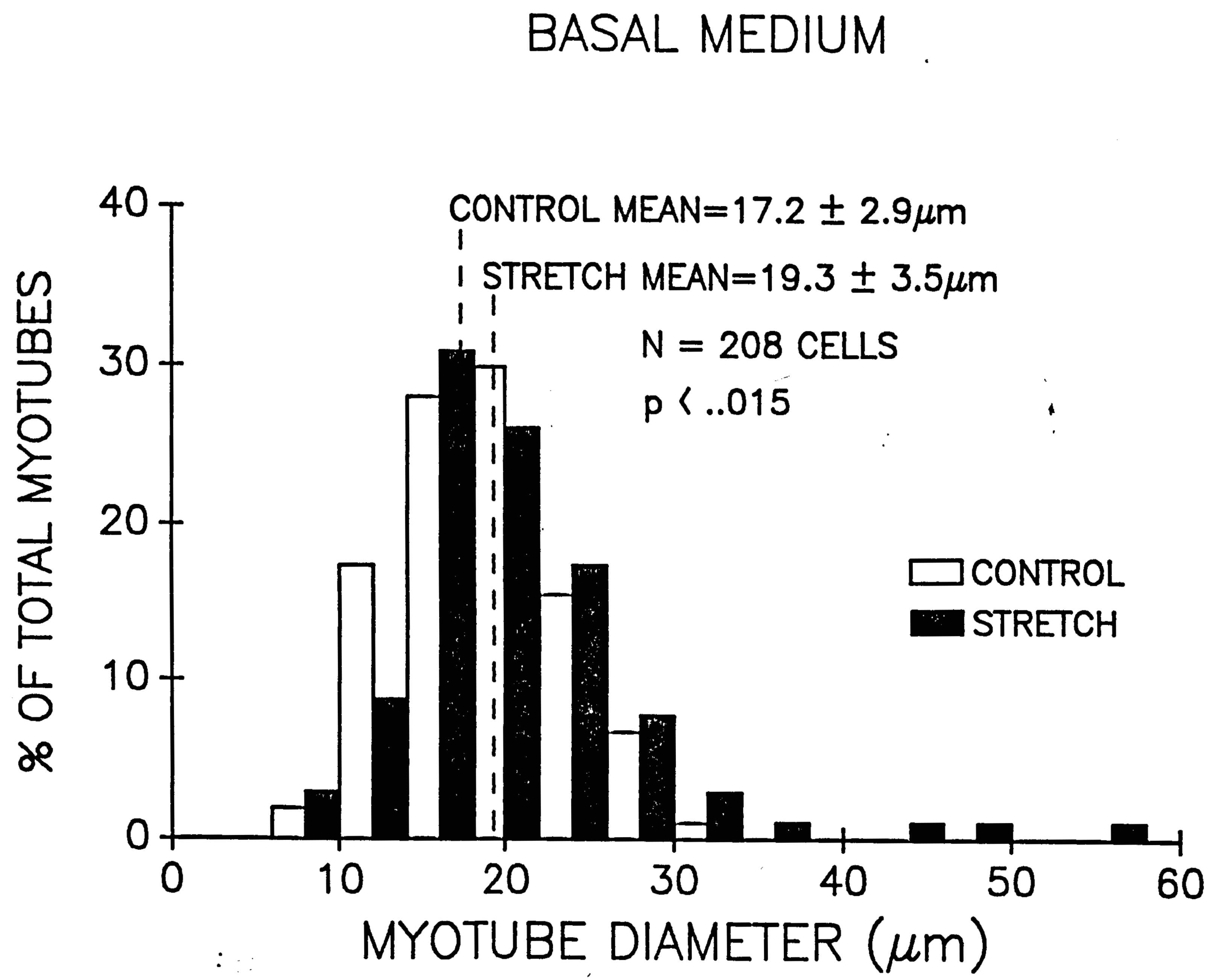
TOTAL DNA CONTENT

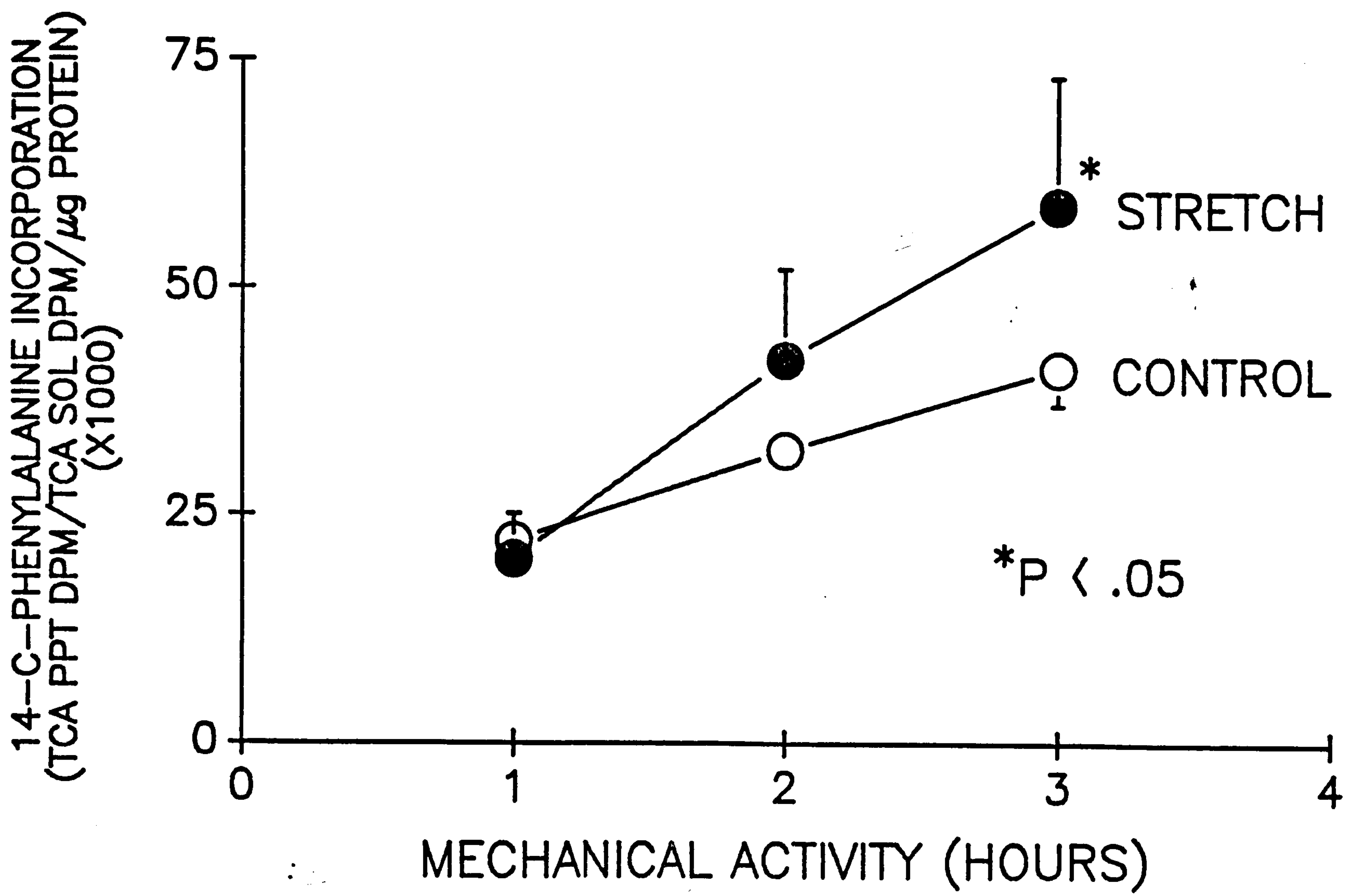


6A

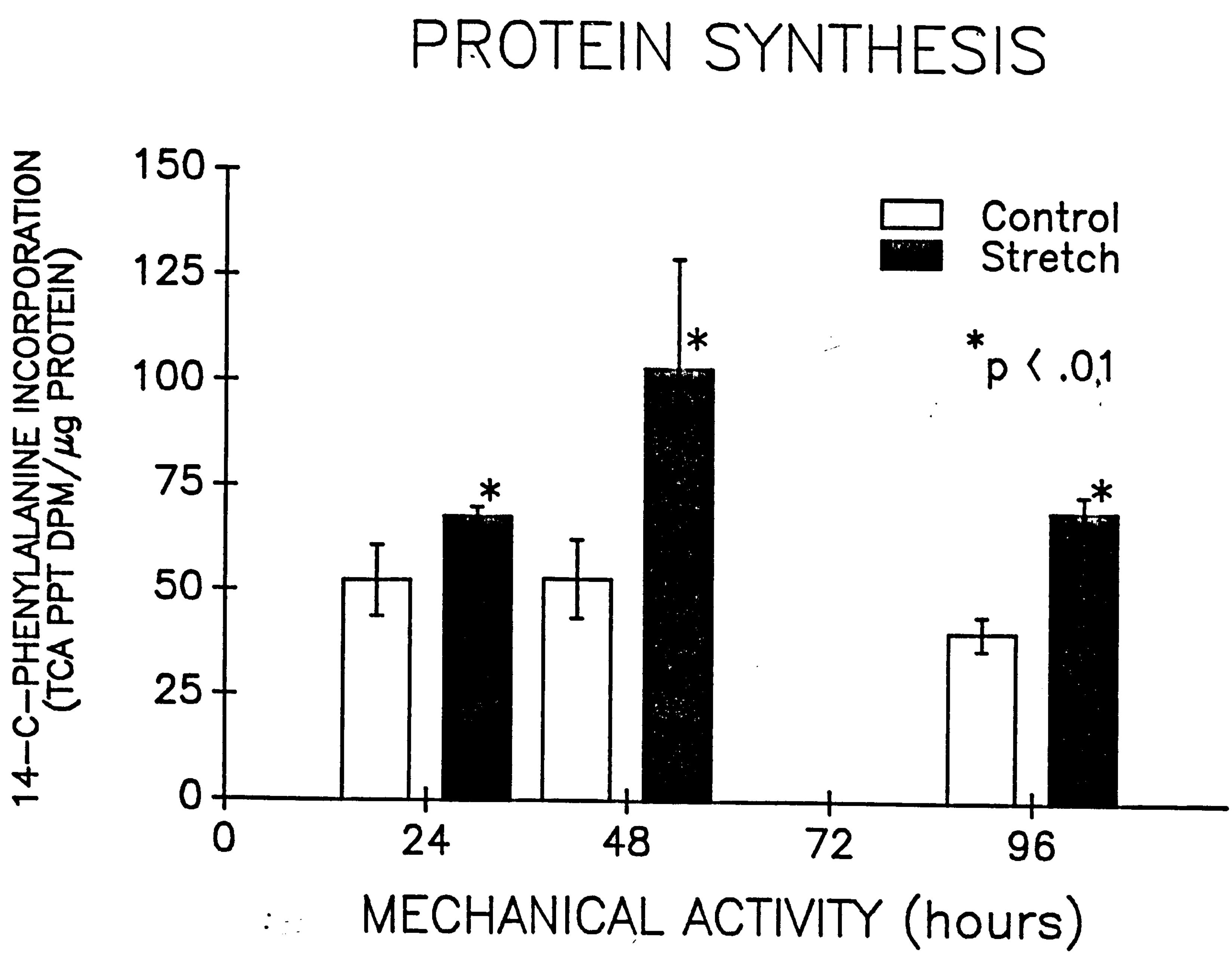


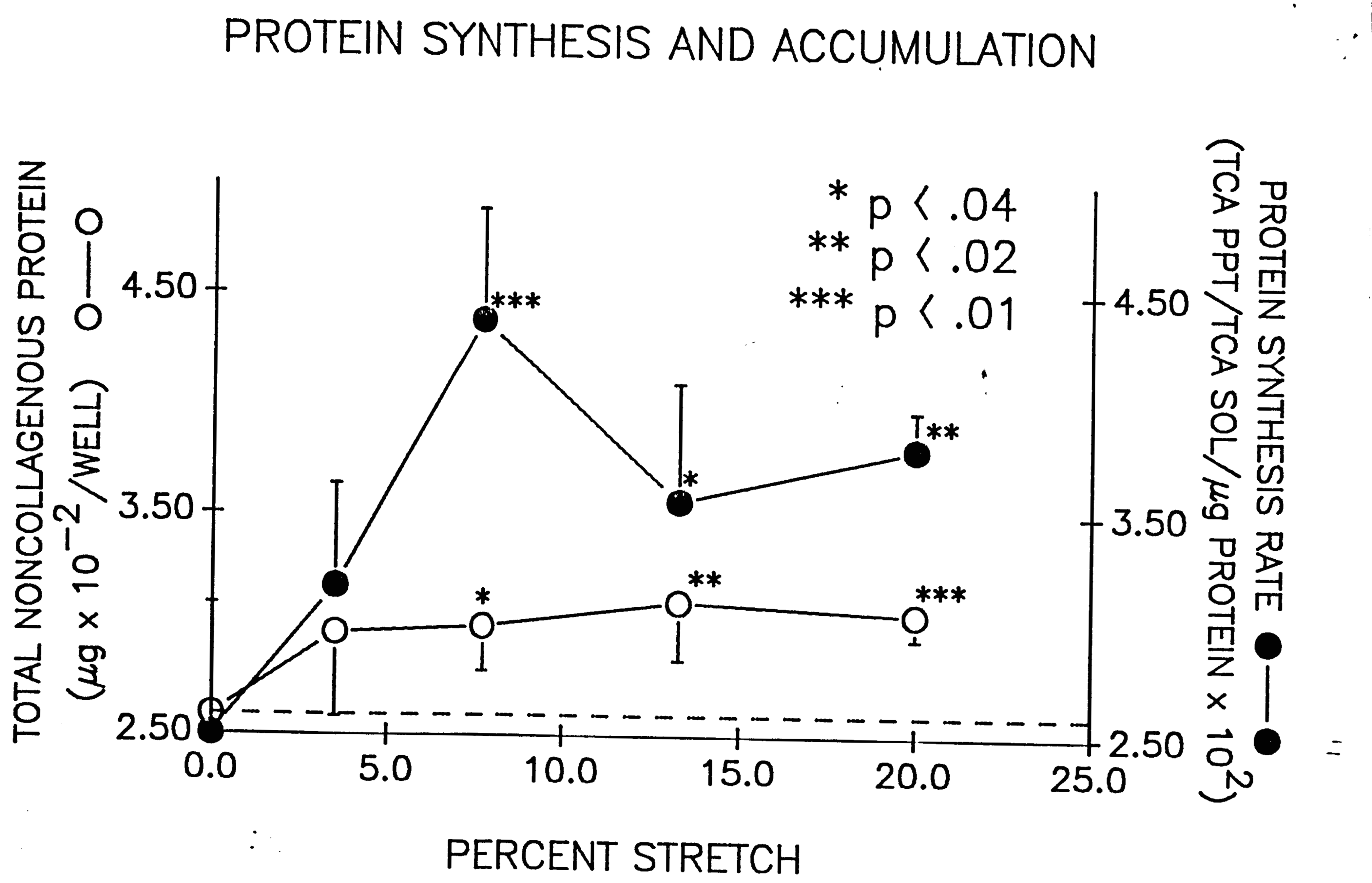
COMPLETE MEDIUM
CONTROL MEAN = 19.2 ± 4.0μm
STRETCH MEAN = 22.3 ± 5.5μm
N = 345 CELLS
p < .003
CONTROL
STRETCH
% OF TOTAL MYOTUBES
0
10
20
30
40
50
MYOTUBE DIAMETER (MICRONS)


BASAL MEDIUM
CONTROL MEAN=17.2 ± 2.9μm
STRETCH MEAN=19.3 ± 3.5μm
N = 208 CELLS
p < ..015
CONTROL
STRETCH
% OF TOTAL MYOTUBES
0
10
20
30
40
0
10
20
30
40
50
60
MYOTUBE DIAMETER (μm)



14–C–PHENYLALANINE INCORPORATION
(TCA PPT DPM/TCA SOL DPM/μg PROTEIN)
(X1000)
75
50
25
0
STRETCH
CONTROL
*P < .05
0
1
2
3
4
MECHANICAL ACTIVITY (HOURS)


PROTEIN SYNTHESIS
14-C-PHENYLALANINE INCORPORATION
(TCA PPT DPM/μg PROTEIN)
150
125
100
75
50
25
0
Control
Stretch
*p < .01
*
0
24
48
72
96
MECHANICAL ACTIVITY (hours)



PROTEIN SYNTHESIS AND ACCUMULATION
TOTAL NONCOLLAGENOUS PROTEIN
(μg x 10^-2/WELL)
PROTEIN SYNTHESIS RATE
(TCA PPT/TCA SOL/μg PROTEIN x 10^2)
* p < .04
** p < .02
*** p < .01
4.50
3.50
2.50
0.0
5.0
10.0
15.0
20.0
25.0
PERCENT STRETCH